Note

Inhibitory Effect of β-Carotene and Astaxanthin on Photosensitized Oxidation of Phospholipid Bilayers

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Summary Large unilamellar liposomes comprising of egg yolk phosphatidylcholine (PC) was exposed to photoirradiation in the presence of methylene blue (water-soluble photosensitizer) or 12-(1-pyrene)dodecanoic acid (P-12, lipid-soluble photosensitizer) to estimate the inhibitory effect of β-carotene and astaxanthin on photosensitized oxidation of phospholipid bilayers. Without sensitizers, astaxanthin decreased much slower than β-carotene and other hydrocarbon carotenoids (lycopene, α-carotene). Astaxanthin lasted longer than β-carotene even in the presence of methylene blue or P-12. Decrease of astaxanthin was also much slower than that of β-carotene when egg yolk PC was replaced by dimyristoyl PC. However, inhibitory effect of astaxanthin was lower than β-carotene in the case of P-12 sensitized photooxidation. These results suggest that effectiveness of carotenoids as antioxidants on photosensitized oxidation (Type II) in phospholipid bilayers depends on the site of singlet oxygen to be generated, as well as their stability on photoirradiation.

Key Words carotenoids, β-carotene, astaxanthin, photosensitized oxidation, singlet oxygen, unilamellar liposomes

Lipid peroxidation in biomembranes has attracted much attention in relation to oxidative damage leading to a wide variety of pathological conditions (1). It has also become apparent that the human body possesses a well-organized cellular defense system against membrane lipid peroxidation (2). This system involves low molecular weight antioxidants such as α-tocopherol and ascorbic acid (3). Carotenoids seem to participate in this defense system because carotenoids can act as chain-breaking antioxidants which interfere the chain reaction of lipid peroxidation by trapping chain-propagating lipid peroxyl radicals (4–6). On the other hand,
carotenoids are well-known singlet oxygen ($^1\text{O}_2$) quenchers (7). $^1\text{O}_2$ reacts with unsaturated lipids resulting in lipid hydroperoxides (8). This reaction seems to be, at least partly, responsible for membrane lipid peroxidation because $^1\text{O}_2$ is found in a variety of biological systems (9). Thus, carotenoids may serve as preventive antioxidants when $^1\text{O}_2$ oxygenation contributes to the initiation of lipid peroxidation. DiMascio et al. (10) have shown that lycopene is the most efficient $^1\text{O}_2$ quencher among carotenoids by calculating their $^1\text{O}_2$ quenching rate constants in solution. However, little is known about the inhibitory effect of each carotenoid on $^1\text{O}_2$-mediated lipid peroxidation in biomembranes.

The purpose of this study is to assess the effectiveness of carotenoids as antioxidants on $^1\text{O}_2$ mediated lipid peroxidation occurring in biomembranes. We selected β-carotene and astaxanthin as a non-polar hydrocarbon carotenoid and a polar oxygenated carotenoid, respectively. Photosensitized oxidation of large unilamellar liposomes with water-soluble photosensitizer or lipid-soluble photosensitizer was examined to generate $^1\text{O}_2$ in this biomembrane model.

Materials and methods. Reagents: β-Carotene, α-carotene, and lycopene were obtained from Sigma Chemicals (St. Louis, MO, USA). Astaxanthin was kindly provided by Hoffmann-La Roche (Basle, Switzerland). d-α-Tocopherol was the gift from Eisai Co. (Tokyo, Japan). Egg yolk phosphatidylycholine (PC) and dimyristoyl PC were products of Sigma Chemicals, and egg yolk PC was purified to remove contaminant peroxides by reverse-phase column chromatography. The purified PC was kept in chloroform solution at $-20^\circ\text{C}$ until it was used in the experiment. Methylene blue was purchased from Kanto Chemical Co. (Tokyo, Japan), and 12-(1-pyrene)dodecanoic acid (P-12) was the product of Lambda Probes & Diagnostic (Graz, Austria).

Preparation of unilamellar liposomes: The chloroform solutions of PC and carotenoids or α-tocopherol were mixed in a test tube and solvent was removed with a stream of nitrogen and finally under vacuum. The residue was dispersed in 0.7 ml Tris-HCl buffer (0.01 M, pH 7.4) which contained 0.5 mM diethylenetriamine pentaacetic acid (DTPA) for preventing the prooxidant effect of contaminant metal ions on the reaction (11). The suspension was mixed with a Vortex mixer for 1 min followed by ultrasonic irradiation in a Branson ultrasonifier for 30 s. Large unilamellar liposomes were obtained by extrusion method (12) as described below. The suspension was introduced to LiposoFast apparatus (AVESTIN Co., Ottawa, Canada) and passed through polycarbonate membrane (pore size 100 nm) 21 times. The resulting liposomal suspension was diluted with the same volume of Tris-HCl buffer. Unilamellar liposomes were prepared just before the peroxidation experiment.

Photosensitized oxidation of liposomes: In methylene blue sensitized photooxidation, liposomal suspension (0.5 ml) was mixed with the 0.6 ml of Tris-HCl buffer containing methylene blue. In P-12 sensitized photooxidation, a chloroform solution of P-12 was added to the chloroform solution containing PC and antioxidants before preparation of unilamellar liposomes. Liposomal suspension was...
placed into photoirradiation chamber (Nippon Medical & Chemical Instruments Co., Osaka, Japan). The suspension was photoirradiated by fluorescent lamp (light intensity at the sample: 112 µE·s⁻¹·m⁻²; wavelength: 350-750 nm (13)) at 37°C with continuous shaking. At regular intervals, an aliquot of the suspension was withdrawn and injected into the HPLC column.

**Determination of carotenoids and α-tocopherol**: Carotenoids were quantified by HPLC using a column of TSK gel ODS 80Ts (TOSOH, Japan) with the eluting solvent of isopropanol/acetonitrile/dichloromethane (7:7:2, v/v/v) at the flow rate of 0.7 ml/min for α-, β-carotene and lycopene, methanol/water (95:5, v/v) at the flow rate of 1.0 ml/min for astaxanthin. The effluent was monitored at 450 nm using a Shimadzu SPD-6AV spectrophotometric detector (Shimadzu, Kyoto, Japan). For the determination of α-tocopherol, a column of Inertsil ODS (GL Science) was used with the eluting solvent of methanol-formic acid (99:1, v/v) containing 30 mM lithium acetate. A amperometric detector ICA-3060 (Toa Electronics Ltd., Tokyo, Japan) was used to monitor the oxidation potential of α-tocopherol at 600 mV. The flow rate was set at 1.2 ml/min.

**Determination of PC-OOH**: PC-OOH were determined by a reversed-phase HPLC according to the method described previously (14). The mixture of methanol and water (95:5, v/v) was used as an eluting solvent and PC-OOH were detected by ultraviolet absorption at 235 nm. The standard PC-OOH was obtained from the oxidized PC by the procedure described previously (15).

**Results**. Figure 1 shows the loss of carotenoids and that of α-tocopherol.

![Graph](image-url)
during the photoirradiation of liposomal suspension without sensitizer. Lycopene was the most susceptible to the photoirradiation among the carotenoids used here. Astaxanthin was resistant to photoirradiation and 40.5% of initial amounts still remained after 48 h incubation. Stability of \( \alpha \)-tocopherol was evidently lower than that of astaxanthin and slightly higher than that of \( \alpha \)- and \( \beta \)-carotene. In the absence of photosensitizer, less than 20 \( \mu \text{M} \) of PC-OOH was found to accumulate after 6 h of the irradiation period (data are not shown here). However, PC-OOH accumulated linearly in the presence of methylene blue and reached to 300 \( \mu \text{M} \) after the irradiation for 3 h (Fig. 2). \( \alpha \)-Tocopherol was consumed much faster than \( \beta \)-carotene and thereby its inhibitory effect was terminated immediately as compared with that of \( \beta \)-carotene. Astaxanthin scarcely decreased during the irradiation. When unilamellar liposomes were exposed to photoirradiation in the presence

![Fig. 2. Loss of antioxidants (A) and accumulation of PC-OOH (B) by methylene blue-sensitized photooxidation of unilamellar liposomes. The reaction system consisted of egg yolk PC (5 mM), antioxidants (5 \( \mu \text{M} \)), methylene blue (100 \( \mu \text{M} \)), DTPA (0.5 mM) in 0.01 M Tris-HCl buffer (1.1 ml, pH 7.4). ○, no addition; ●, \( \beta \)-carotene; □, astaxanthin; †, \( \alpha \)-tocopherol.](image)

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of P-12, α-tocopherol disappeared faster than astaxanthin and β-carotene resulting in the little effect on PC-OOH accumulation (Fig. 3). Astaxanthin decreased slower than β-carotene. However, the inhibitory effect of astaxanthin was apparently lower than that of β-carotene. Figure 4 shows the decreases of β-carotene and astaxanthin when liposomes comprising of dimyristoyl PC was photoirradiated in the presence of methylene blue or P-12. In both cases, β-carotene decreased much faster than astaxanthin.

Discussion. Photosensitized oxidation is categorized to free radical-driven reaction (Type I) and 1O2 oxygenation (Type II). There are enough evidences that both methylene blue and P-12 sensitized photooxidation of unsaturated lipids proceed via Type II process (16). Antioxidants inhibit 1O2 oxygenation through physical quenching (scheme 1) or chemical reaction (scheme 2) (17).

![Graph A](image1)

![Graph B](image2)

Fig. 3. Loss of antioxidants (A) and accumulation of PC-OOH (B) by P-12 sensitized phooxidation of unilamellar liposomes. The reaction system consisted of egg yolk PC (5 mM) and antioxidants (5 µM), P-12 (1 mM), DTPA (0.5 mM) in 0.01 M Tris-HCl buffer (1.1 ml, pH 7.4). ○, no addition; ●, β-carotene; □, astaxanthin; +, α-tocopherol.
Fig. 4. Loss of \( \beta \)-carotene and astaxanthin by methylene blue (A) and P-12 (B) sensitized photooxidation in dimyristoyl PC liposomes. The reaction system consisted of dimyristoyl PC (5 mM) and antioxidant (5 \( \mu \)M), methylene blue (100 \( \mu \)M) or P-12 (1 mM), and DTPA (0.5 mM) in 0.01 M Tris-HCl buffer (1.1 ml, pH 7.4). ●, \( \beta \)-carotene; □, astaxanthin.

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\begin{align*}
^1\text{O}_2 + A &\rightarrow \ ^3\text{O}_2 + A, \quad (1) \\
^1\text{O}_2 + A &\rightarrow \text{AO}_2. \quad (2)
\end{align*}
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A: antioxidant.

Physical quenching of \( ^1\text{O}_2 \) takes a major part in \( ^1\text{O}_2 \) quenching activity of carotenoids and no carotenoids change chemically after physical quenching (7). However, carotenoids are oxidized by chemical reaction with \( ^1\text{O}_2 \). In addition, carotenoids are susceptible to photoirradiation as shown in Fig. 1. Thus, carotenoids are consumed by photoirradiation itself and chemical reaction with \( ^1\text{O}_2 \) during photosensitized oxidation.

It is known that the physical quenching rate constants by \( \beta \)-carotene are ca. 100 times higher than that of \( \alpha \)-tocopherol (18). \( \beta \)-Carotene consumed slower than...
α-tocopherol in both of methylene blue and P-12 sensitized photooxidation (Figs. 2, 3), although β-carotene was more susceptible to the photoirradiation itself (Fig. 1). It is therefore likely that β-carotene is more resistant to chemical reaction with 1O₂ than α-tocopherol and thereby its inhibitory effect lasts longer than α-tocopherol in photosensitized oxidation of membrane phospholipids.

Astaxanthin was more resistant to photoirradiation itself than hydrocarbon carotenoids including β-carotene as shown in Fig. 1. In addition, astaxanthin was also much stable against photosensitized oxidation in dimyristoyl PC (Fig. 4). It is therefore likely that chemical reaction of this polar carotenoid with 1O₂ is slower than that of β-carotene. It has been shown that astaxanthin exhibits the physical quenching rate constant comparable to that of β-carotene (10). Thus, slower consumption of astaxanthin may lead to effective inhibition of 1O₂ oxidation in membrane phospholipids. Miki (19) indicated that astaxanthin was an extremely powerful antioxidant on methylene blue sensitized photooxidation of methyl linoleate in emulsion. Nevertheless, the fact that the antioxidative effect of astaxanthin was lower than β-carotene in P-12 sensitized photooxidation (Fig. 3) implies that the site of 1O₂ to be generated and the location of carotenoids in phospholipid bilayers are of much importance to assess the 1O₂ quenching activity in biomembranes. It is probable that this lipid-soluble sensitizer generates 1O₂ within the membranes. Astaxanthin is likely to be oriented to the parallell to the hydrocarbon chain of phospholipids in the bilayer structure while nonpolar carotene is favored to orient its long isoprenoid chain perpendicular to the lipid acyl group (20, 21). We found that inhibitory effect of α-carotene was almost the same as that of β-carotene, as expected from its structural similarity (data are not shown here). Therefore, the difference of the orientation in the bilayers may explain the superiority of β-carotene than astaxanthin as an antioxidant on P-12 sensitized photooxidation.

Long-term photoirradiation of phospholipid bilayers may cause free radical chain oxidation of phospholipids and cooxidation of carotenoids by generating free radicals from preformed phospholipid hydroperoxides. However, we previously demonstrated that the activity of carotenoids as chain-breaking antioxidants in liposomal suspension was much lower than that of α-tocopherol under atmospheric oxygen (22) and low partial pressure of oxygen (23). Therefore, the inhibition of 1O₂ oxygenation is mainly responsible for the antioxidant activity of carotenoids on photosensitized oxidation (Type II) in phospholipid bilayers.

In conclusion, site of 1O₂ to be generated and the location of carotenoids in phospholipid bilayers should be taken into account to assess the inhibitory effect on 1O₂ oxygenation of biomembranes.

REFERENCES


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